fermentation

MDPI

Article

Fed-Batch Bioreactor Cultivation of *Bacillus subtilis* Using Vegetable Juice as an Alternative Carbon Source for Lipopeptides Production: A Shift towards a Circular Bioeconomy

Irene Gugel [1], Maliheh Vahidinasab [2,*], Elvio Henrique Benatto Perino [2], Eric Hiller [2], Filippo Marchetti [1], Stefania Costa [1,3,*], Jens Pfannstiel [4], Philipp Konnerth [5], Silvia Vertuani [1], Stefano Manfredini [1] and Rudolf Hausmann [2]

[1] Department of Life Sciences and Biotechnology, University of Ferrara, 44121 Ferrara, Italy; silvia.vertuani@unife.it (S.V.)
[2] Department of Bioprocess Engineering (150k), Institute of Food Science and Biotechnology, University of Hohenheim, Fruwirthstrasse 12, 70599 Stuttgart, Germany; eperino@uni-hohenheim.de (E.H.B.P.)
[3] Department of Chemical, Pharmaceutical and Agrarian Sciences, University of Ferrara, 44121 Ferrara, Italy
[4] Core Facility Hohenheim, Mass Spectrometry Unit, University of Hohenheim, Ottlie-Zeller-Weg 2, 70599 Stuttgart, Germany
[5] Department of Conversion Technology of Biobased Resources, University of Hohenheim, Garbenstrasse 9, 70599 Stuttgart, Germany; philipp.konnerth@uni-hohenheim.de
* Correspondence: malihe.vahidinasab@uni-hohenheim.de (M.V.); stefania.costa@unife.it (S.C.)

Abstract: In a scenario of increasing alarm about food waste due to rapid urbanization, population growth and lifestyle changes, this study aims to explore the valorization of waste from the retail sector as potential substrates for the biotechnological production of biosurfactants. With a perspective of increasingly contributing to the realization of the circular bioeconomy, a vegetable juice, derived from unsold fruits and vegetables, as a carbon source was used to produce lipopeptides such as surfactin and fengycin. The results from the shake flask cultivations revealed that different concentrations of vegetable juice could effectively serve as carbon sources and that the fed-batch bioreactor cultivation strategy allowed the yields of lipopeptides to be significantly increased. In particular, the product/substrate yield of 0.09 g/g for surfactin and 0.85 mg/g for fengycin was obtained with maximum concentrations of 2.77 g/L and 27.53 mg/L after 16 h, respectively. To conclude, this study provides the successful fed-batch cultivation of *B. subtilis* using waste product as the carbon source to produce secondary metabolites. Therefore, the consumption of agricultural product wastes might be a promising source for producing valuable metabolites which have promising application potential to be used in several fields of biological controls of fungal diseases.

Keywords: surfactin; fengycin; lipopeptides; secondary metabolite; biosurfactant; circular bioeconomy; fed-batch fermentation

check for updates

Citation: Gugel, I.; Vahidinasab, M.; Benatto Perino, E.H.; Hiller, E.; Marchetti, F.; Costa, S.; Pfannstiel, J.; Konnerth, P.; Vertuani, S.; Manfredini, S.; et al. Fed-Batch Bioreactor Cultivation of *Bacillus subtilis* Using Vegetable Juice as an Alternative Carbon Source for Lipopeptides Production: A Shift towards a Circular Bioeconomy. *Fermentation* **2024**, *10*, 323. https://doi.org/10.3390/fermentation10060323

Academic Editor: Eduardo Gudiña

Received: 6 May 2024
Revised: 15 June 2024
Accepted: 17 June 2024
Published: 20 June 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Due to rapid population growth and urbanization, coupled with industrial development and changing lifestyles, food waste (FW) is now a global challenge [1]. Food waste, as defined by the Food and Agriculture Organization (FAO), refers to food fit for human consumption that has been discarded, regardless of whether it has passed its sell-by date or has deteriorated [2]. Approaching the issue of food waste, the problem lies not only on an economic and ethical level but also on an environmental level [3]. Landfilling is one of the oldest and most traditional ways of dealing with FW. However, this method of disposal contributes to global greenhouse gas emissions and consequently to climate change [1,4–6].

According to UNEP's Food Waste Index Report, 931 million tons of food were generated in 2019, accounting for 17% of the global food production [7]. In particular, more than 58 million tons of food waste are generated each year in the European Union, corresponding

to 131 kg of food waste per capita [8]. This staggering amount of food waste not only represents a significant loss of resources but also has considerable economic and environmental implications. The Food and Agriculture Organization (FAO) has reported that the global full costs of food wastage amount to about USD 2.6 trillion per year, encompassing USD 700 billion in environmental costs and USD 900 billion in social costs [9]. This highlights the critical need for effective food waste management strategies along the entire food supply chain, where retail plays a significant role [10]. At this level, fresh fruit and vegetables were identified as the main retail food waste [11,12]. In this respect, Scholz et al., taking six stores over a period of three years, found that 85% of the wasted mass was fruit and vegetables, totaling 1340 tons [13]. Although in the waste management hierarchy the 'prevention' option is the most desirable, thinking about the reuse of unavoidable FW is an appropriate option for addressing the food waste challenge [14]. Fruit and vegetable wastes contain various free sugars, such as glucose, fructose and sucrose, which can be used as carbon sources for bacterial fermentation [15]. They therefore represent a potential feedstock that can be efficiently exploited for the biotechnological production of bio-based chemicals [16–18]. The efficient use of resources as well as the reduction in environmental impact are the basis for the development of waste biorefineries. In this context, the concepts of circular economy and bioeconomy converge, giving rise to the emerging term 'circular bioeconomy', which challenges the conversion of waste biomass into high-value chemicals [6,19,20].

In recent decades, biosurfactants have been recognized as one of the most promising classes of molecules due to their wide range of applications [21]. It has been estimated that the global biosurfactant market will grow to USD 1.4427 billion in 2026 [22]. In this context, lipopeptides such as surfactin and fengycin, originally identified as secondary metabolites produced by *Bacillus subtilis*, are one group of the most effective biosurfactants reported to date [23]. These are compounds characterized by a lipophilic fatty acid chain and a hydrophilic peptide ring. Due to their amphiphilic structure, lipopeptides possess several bioactivities such as antibacterial, antifungal, antiviral and antitumor activities, and therefore, they are of high interest for the chemical, agricultural, pharmaceutical and food industries [24–26]. Moreover, thanks to their remarkable surface properties, they are increasingly of interest in the cosmetic field [27]. Interestingly, various wastes have recently been studied, e.g., date molasses [28], white bean powder [29], brewery waste [30] and corn steep liquor [31], as a carbon source for *Bacillus* growth and lipopeptide production. By using brewery waste, a maximum biosurfactant titer of 657 mg/L was obtained, whereas a higher titer was reached with white bean powder and date molasses (1.17 g/L and 2.29 g/L of surfactin, respectively). Furthermore, by adding corn steep liquor with micronutrients such as Mn^{2+}, Mg^{2+} and Fe^{2+}, the amount of surfactin produced was increased to 4.8 g/L. This interest in finding suitable waste substrates is explained by the fact that the production cost of a biosurfactant is currently higher than that of synthetic products, and the use of waste as a substrate can significantly reduce the overall process costs [30,32].

Furthermore, although the alternative above-mentioned substrates have proven their worth, they have only been tested in flask cultivation, and more efficient fermentation strategies, such as fed-batch cultivation, remain largely unexplored for most of the waste substrates studied so far in the scientific literature.

B. subtilis, belonging to the genus *Bacillus*, has a fully sequenced genome that has efficient secretion pathways for the transport of secondary metabolites in the extracellular space. For these reasons, *B. subtilis* lends itself to genetic engineering studies [32].

Recently, Vahidinasab et al. modified the *Bacillus subtilis* 3NA to obtain the *sfp*+ derivative (BMV9), which showed good surfactin and fengycin biosynthesis capabilities when cultivated in Mineral Salt Medium (MSM) using glucose as a carbon source [33].

Whole genome sequencing of *B. subtilis* 3NA is available, and it is characterized as a hybrid strain with genetic features of the model strains *B. subtilis* 168 and W23. Strain 3NA is known as a non-sporulation strain suitable for the high-cell-density fed-batch cultivation of bacteria due to a mutation in the *spo0A* gene [34]. However, the strain BMV9 has not yet been investigated for the production of lipopeptides using an alternative growth

medium, such as waste carbon sources, thereby proposing a novel approach to biowaste utilization. Therefore, this study aimed to evaluate the ability of *B. subtilis* BMV9 to grow and produce lipopeptides using an alternative growth medium obtained from unsold fruit and vegetables from a local supermarket (vegetable juice, VJ). In addition, the efficacy of a fed-batch fermentation strategy in enhancing the lipopeptide yield and reducing the fermentation time, aligning with sustainability goals, has been investigated. Finally, the lipopeptides obtained from the FW waste medium were characterized by LC-MS.

2. Materials and Methods

2.1. Bacterial Strain and Media

The strain used in this study was *Bacillus subtilis* BMV9 [33]. The Mineral Salt Medium (MSM) used to carry out flask cultivation tests was described by Wenzel et al. [35] and it consisted of 1 g/L $(NH_4)_2$-H-citrate, 2 g/L Na_2SO_4, 2.68 g/L $(NH_4)_2SO_4$, 0.5 g/L NH_4Cl, 14.6 g/L K_2HPO_4, 4 g/L $NaH_2PO_4 \times H_2O$, 1 g/L $MgSO_4 \times 7\ H_2O$ and 3 mL/L Trace Elements Solution (TES). The trace element solution consisted of 10.05 g/L Na_2EDTA, 0.5 g/L $CaCl_2$, 8.35 g/L $FeCl_3$, 0.1 g/L $MnSO_4 \times H_2O$, 0.18 g/L $ZnSO_4 \times 7\ H_2O$, 0.16 g/L $CuSO_4 \times 5\ H_2O$ and 0.18 g/L $CoCl_2 \times 6\ H_2O$. All chemicals were purchased by Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Standard of surfactin and fengycin were provided by Sigma-Aldrich, St. Louis, MO, USA and Lipofabrik, Lesquin, France respectively. The vegetable juice was supplied by the research and development network "Neues Kreislaufsystem 2 + 12" (Aulendorf, Germany). It consists of a mixture of vegetable and fruit waste collected from German supermarkets. It is likely that the fruit and vegetables had entered a short spontaneous fermentation before being pressed due to the traces of lactic acid within the VJ. The vegetable juice was centrifuged at 10,000× *g* for 30 min to remove the solid particles. The supernatant was collected and used in this study. The exact composition of the vegetable juice used in this study was performed by an external laboratory and is shown in Table 1.

Table 1. Composition of vegetable juice.

Parameter	Concentration
Total nitrogen (g/100 g)	0.90 ± 0.10
Fructose (g/100 g)	2.89 ± 0.28
Glucose (g/100 g)	1.96 ± 0.23
Sucrose (g/100 g)	0.12 ± 0.02
Mineral composition (mg/kg):	
Copper	0.274 ± 0.107
Zinc	0.596 ± 0.206
Calcium	112 ± 15
Iron	0.972 ± 0.312
Magnesium	43.10 ± 5.60
Potassium	1060 ± 140
Manganese	1.13 ± 0.35
Organic acids (g/L):	
Maleic acid	0.914 ± 0.01
Lactic acid	0.089 ± 0.03
Formic acid	0.089 ± 0.01

Sugars analysis was performed following the Accredited Test Procedure: 07(S46) Rev. 10 2012-GC-FID. The nitrogen content was determined by the Kjeldahl method. The mineral composition was investigated following the Accredited Test Procedures 05(ICP-MS) 2021 Rev.4 for copper, zinc, iron and manganese and 05(ICP-OES) 2019 Rev.2 for calcium, magnesium and potassium. The organic acids were identified with a column BioRad Aminex HPX-87H, 300 × 7.8 mm (Bio-Rad Laboratories, Inc. Hercules, CA, USA) in a Merck Hitachi Primade HPLC system. As an eluent, bi-distilled and degassed water was

diluted with sulfuric acid (4 mM). The column was operated at 25 °C with a flow rate of 0.65 mL min^{-1}.

2.2. Shake Flask Cultivation

The effect of the vegetable juice concentration (VJ) on the lipopeptides production by *B. subtilis* BMV9 was studied in shake flask cultures using 25%, 45%, 70% and 90% VJ in the fermentation medium (Table 2). MSM salts were added to each medium in the same amounts, but with no additional sugars. MSM added with 20 g/L glucose was used as a control. The pH of each medium was set to 7.

Table 2. Composition of media used in shake flask cultivation.

Medium Composition		
100% MSM	-	100 mL MSM (+20 g/L Glucose)
90% VJ	90 mL of VJ	10 mL dd-water + salts
70% VJ	70 mL of VJ	30 mL dd-water + salts
45% VJ	45 mL of VJ	55 mL dd-water + salts
25% VJ	25 mL of VJ	75 mL dd-water + salts

For the pre-culture preparation, 10 µL of *B. subtilis* BMV9 glycerol stock solution was used to inoculate 10 mL LB broth (10 g/L Tryptone, 5 g/L Yeast extract, 5 g/L NaCl) in a baffled flask, which were incubated overnight at 37 °C and 120 rpm. After the incubation period, the bacterial suspension was used to inoculate each media to an initial OD_{600} of 0.1. The main cultivations were carried out in 1L baffled flasks with a working volume of 100 mL at 37 °C and 120 rpm for 48 h. Samples were taken every 3 h during the fermentation process. The shake flask cultivations were performed in triplicate.

2.3. Fed-Batch Cultivation

For the fed-batch cultivation, the first pre-culture was obtained by inoculating 10 mL of LB with 10 µL of *B. subtilis* BMV9 glycerol stock solution. After 12 h of incubation at 37 °C and 120 rpm, it was used to inoculate the second pre-culture (100 mL of 25% VJ broth, OD_{600} of 0.1), which was incubated in the same conditions for 10 h to reach the log phase. This bacterial suspension was used as an inoculum for fed-batch fermentation with a starting working volume of 5-L of 25% VJ medium and a starting OD_{600} of 0.1.

Cultivation was carried out in a 10 L bioreactor (ZETA GmbH, Graz/Lieboch, Austria) at 37 °C with a stirring rate of 300 rpm using Rushton turbines. Dissolved oxygen levels were maintained at a minimum of 50% by using an internal probe, through the adjustment of the stirrer speed and aeration rate. The values of pH were kept constant to 7 (feeding 20% (*v*/*v*) NH_3) by using an internal probe. The flow rate was 10 NL/min. Samples were taken every 2 h during the batch phase. When the carbon substrates were completely depleted, the fed-batch phase was started by exponentially pumping the 2 L Feed solution consisting of undiluted VJ, 12 g L^{-1} $MgSO_4$ and 120 mL L^{-1} TES. The feed rate was calculated using the equation reported by Klausmann et al. [36].

Since the calculation of the initial feed rate is linked to the biomass reached at the end of the batch phase, this value was calculated specifically for each fed-batch cultivation.

Samples were taken every hour until the feed was depleted. As methods of protection against overfoaming, a foam centrifuge, a foam trap in front of the exhaust gas filter and the antifoam agent Contraspum A4050 (Zschimmer & Schwarz GmbH, Lahnstein, Germany) were used. Three independent experiments were performed.

2.4. Lipopeptides Extraction

Samples from the shake flask cultivation were centrifuged for removing the biomass. The cell-free supernatant was mixed three times with the extraction solvent, consisting of chloroform/methanol (2:1, *v*/*v*). Then, the mixture was vortexed for 1 min and centrifuged at 3000 rpm for 5 min to facilitate the phase separation. The organic phases were collected

and evaporated under vacuum at 45 °C and 10 mbar for 1 h. Once the organic phases were evaporated, the residues were solubilized in methanol and further analyzed by HPTLC to quantify surfactin and fengycin [33].

2.5. LC-MS/MS Analyses of Lipopeptides

LC-MS/MS analyses of lipopeptides were performed as described previously [37], with minor modifications. In brief, LC-MS/MS analyses were performed on a 1290 UHPLC system (Agilent, Waldbronn, Germany) coupled to a Q-Exactive Plus Orbitrap mass spectrometer source (Thermo Fisher Scientific, Bremen, Germany). Analyte separation was achieved by an ACQUITY CSH C18 column (1.7 µm, 2.1 µm × 150 mm, Waters, Eschborn, Germany). The samples were analyzed in triplicate, and 15 µL of each sample was injected. Mobile phase A was 0.2% formic acid in water, and mobile phase B was 0.2% formic acid in acetonitrile using a constant flow rate of 0.3 mL/min.

The HESI source was operated in positive ion mode with a spray voltage of 4.20 kV. Mass spectra were acquired within the mass range of 500 to 1700 m/z at a resolution of 70,000 FWHM using an Automatic Gain Control (AGC) target of 5.0×10^6 and a 200 ms maximum ion injection time (MIT). Data-dependent MS/MS spectra in a mass range of 50 to 1500 m/z were acquired for the five most abundant precursor ions with the following settings: fixed first mass 50 m/z, resolution 17,500 FWHM, AGC target 5.0×10^6, 200 ms MIT and a stepped collision energy of 15, 30 and 45. Xcalibur™ software version 4.4.16.14 (Thermo Fisher Scientific, San Jose, CA, USA) was used for the data acquisition and data analysis. Peak areas of individual lipopeptides were calculated using extracted ion chromatograms (XICs) of the corresponding precursor ions. The assignment of individual lipopeptides was based on the precise m/z value of the precursor ion, the manual inspection of the corresponding MS/MS spectra and a comparison with available MS/MS spectra from the literature [37].

2.6. Other Analytical Analysis

The samples taken were analyzed for OD_{600}, sugars, surfactin and fengycin concentration. OD_{600} was determined spectrophotometrically. Uninoculated VJ at the same concentration as the tested media was used as a blank. Surfactin and fengycin were analyzed with an HPTLC system (CAMAG, Muttenz, Switzerland) using a validated method, as described previously [38]. Glucose, fructose and sucrose concentrations were determined by using a specific enzymatic kit from R-Biopharm (Darmstadt, Germany), carefully following the manufacturer's instructions. Data were reported as the mean value ± SD.

2.7. Data Analysis

The yield product per biomass ($Y_{p/x}$) [Equation (1)] and product per substrate ($Y_{p/s}$) [Equation (2)] were calculated at maximum surfactin and fengycin concentrations. The yield biomass per substrate ($Y_{x/s}$) [Equation (3)] and specific growth rate (µ) [Equation (4)] were calculated at a maximum cell dry weight (CDW). For the CDW calculation, the correcting factor of 4.31 was used. The specific productivity ($q_{p/x}$) [Equation (5)] was calculated by dividing $Y_{p/x}$ per hours of fermentation ($time_{lipopeptide\ max} - time_{lipopeptide\ 0}$) [39].

$$Y_{p/x} = \frac{m_{Lipopeptide\ max} - m_{Lipopeptide\ t0}}{m_{CDW_{Lipopeptide\ max}} - m_{CDW_{t0}}} \quad (1)$$

$$Y_{p/s} = \frac{m_{Lipopeptide\ max} - m_{Lipopeptide\ t0}}{\Delta S} \quad (2)$$

$$Y_{x/s} = \frac{m_{CDWmax} - m_{CDW_{t0}}}{\Delta S} \quad (3)$$

$$\mu = \frac{\ln \frac{m_{CDW\ max}}{m_{CDW\ t0}}}{t_{CDW\ max} - t_{CDW\ t0}} \quad (4)$$

$$q_{p/x} = \frac{Y_{p/x}}{\Delta t} \quad (5)$$

3. Results and Discussion

3.1. Shake Flasks Cultivation Using VJ as a Carbon Source

Vegetables and fruits contain three main sugars: fructose, glucose and sucrose, which are actually present in VJ. For this reason, VJ was tested for its potential as a carbon source for microbial lipopeptides production.

At the start of the study, *B. subtilis* BMV9 was cultivated in shake flasks using an MSM with different concentrations of VJ serving as the carbon source in order to identify the optimal concentration of VJ for lipopeptides production.

In Figure 1, where the cultivation patterns are shown, it can be seen that the increased percentage of vegetable juice in the medium corresponds to a higher presence of available sugars. Previously, a study conducted on 13 strains of *B. subtilis* showed that all three sugars (glucose, fructose and sucrose) present in the VJ matrix under study could be metabolized to produce surfactin [40]. As is shown in Figure 1, these sugars are also substrates of *B. subtilis* BMV9 since, in all the flask tests, they were almost completely consumed within 27 h in the culture medium with VJ dilutions of 90% and 70% and within 24 h in the culture medium with VJ dilutions of 45% and 25%. However, in this study, the carbon substrates within the media have been consumed differently by *B. subtilis* BMV9. Sucrose was the most rapidly metabolized sugar, as can be observed in the media at 90 and 70% VJ. It is indeed completely consumed within 12 h, even during the lag, and this trend is confirmed by Nitschke et al., who found in cassava water a more rapid consumption of sucrose than of fructose and glucose by *B. subtilis*, going from 20.0 to 1.13 g/L in 12 h [41]; similarly, in another study, it was observed that in the presence of both glucose and sucrose, *B. subtilis* consumes the latter faster [42].

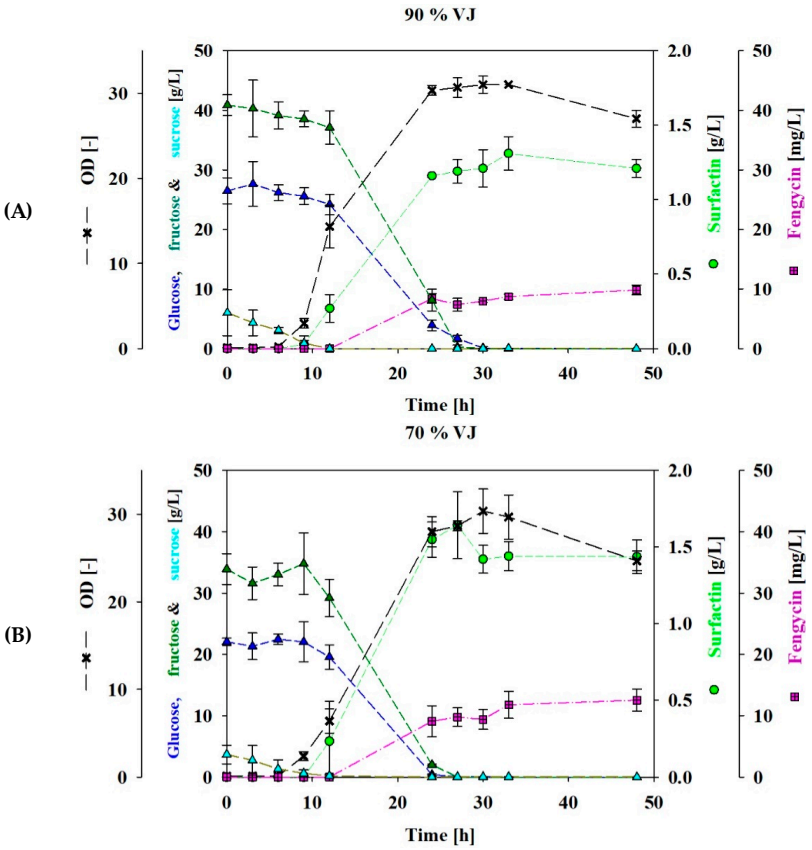

Figure 1. *Cont.*

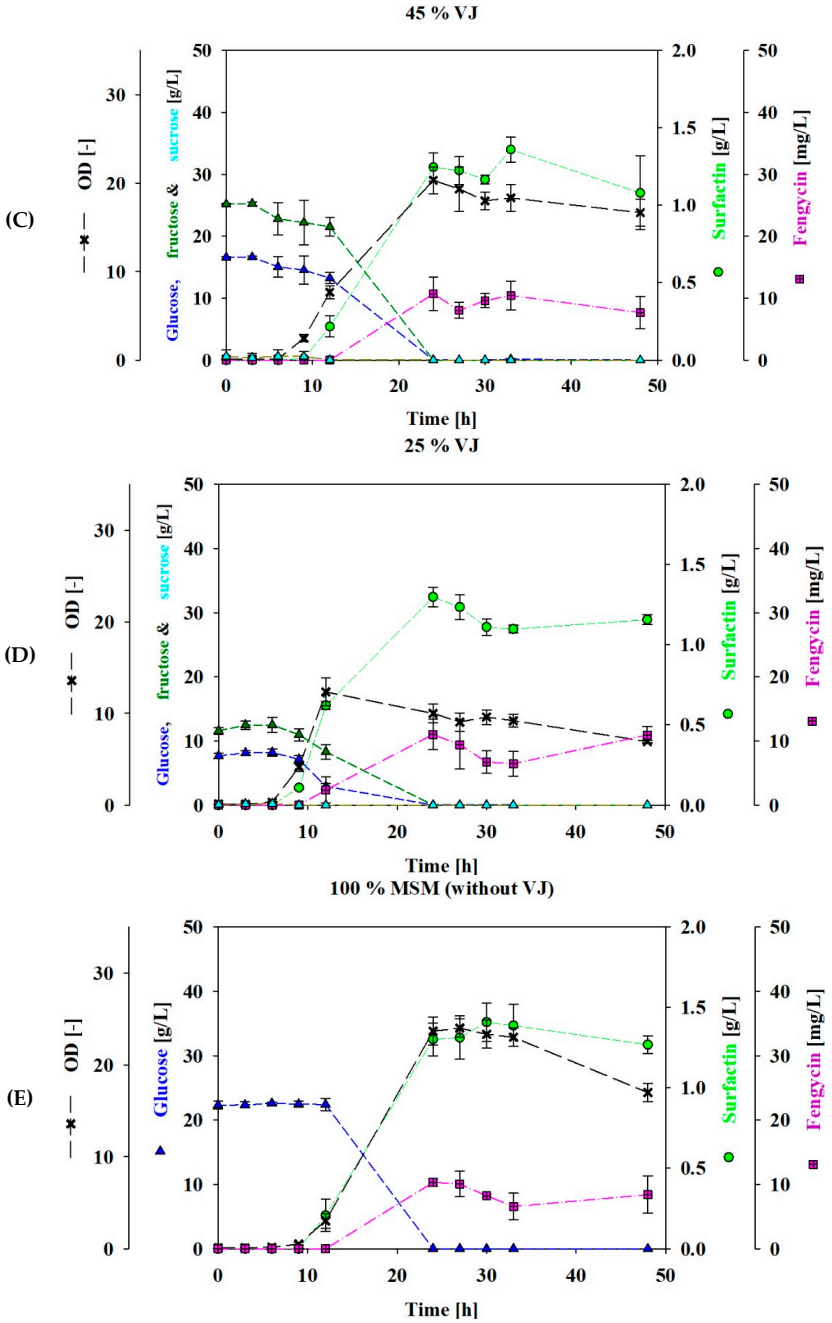

Figure 1. Cultivation pattern of *B. subtilis* BMV9 growing on media composed of different percentages of VJ. The shake flask cultivations were conducted in VJ at a concentration of 90% (**A**), 70% (**B**), 45% (**C**) and 25% (**D**). The same experiment was also conducted using MSM + 20 g/L glucose as a control (**E**).

In media with lower VJ percentages, the dilution effect did not allow the presence of sucrose to be detected (Figure 1C,D). The other two carbon substrates in the media, glucose and fructose, with the exception of a few fluctuations, started to be consumed at the end of the lag phase.

The main difference between media composed of different percentages of food waste is therefore the initial concentration of sugars (glucose, fructose and sucrose), which determines differences in terms of bacterial biomass. Regarding this parameter, as can be seen in Figure 1C, in the 45% VJ medium (41.76 g/L of total starting sugars), the bacteria reached an optical density (OD_{600}) of 20.33 after 24 h of incubation, quite similar to that found after 27 h (OD_{600} = 24) in the MSM control medium using glucose as the carbon source (Figure 1E), despite the fact that, in the latter case, the amount of starting sugars were half

of that in the former (22.31 g/L). In contrast, half of the biomass was unexpectedly found in the 25% VJ medium, even though there were 19.38 g/L of starting sugars in this medium, and we would have expected OD_{600} values comparable to those of the control. However, in the 25% VJ medium, *B. subtilis* reached the maximum OD_{600} (12.33) in only 12 h and then entered the stationary phase, even in the presence of a low concentration of available sugars. In the study conducted by Maliheh et al., the same strain cultured in MSM medium with 20 g/L glucose and 50 mM urea gave similar optical density values but over a much longer period of time of 32 h from the start of fermentation [33].

Increasing the percentage of VJ in the culture medium to 70% (total starting sugars: 59.61 g/L) resulted in an optical density of 30.33 after 30 h of incubation; by increasing the amount of VJ further to 90% and consequently increasing the starting sugars to 73.36 g/L, a similar result (OD_{600} = 31) was obtained in the same time frame. This could be related to the fact that excessive glucose concentrations have been observed to negatively influence the growth behavior of *B. subtilis* [43]. It is therefore possible to state that the decrease in the concentration of the starting sugars in media containing VJ led in turn to a decrease in the producible bacterial biomass.

The detailed bacterial growth curve of *B. subtilis* BMV9 on different VJ media and in MSM is reported in Figure S1.

On the other hand, the reduction in total starting sugars did not affect the total production of lipopeptides by *Bacillus subtilis* BMV9. As a matter of fact, in all the graphs shown in Figure 1, it can be seen that the production of the two lipopeptides is almost equivalent in all media, including the control medium.

Regarding the fermentation trend, in the medium consisting exclusively of MSM, *B. subtilis* started to produce surfactin after 8 h of incubation, and production continues throughout the exponential growth phase, stopping at the beginning of the stationary phase. Unlike surfactin, fengycin was produced shortly after the bacterium entered the log phase, i.e., after 12 h. However, its production did not continue with the onset of the stationary phase of *B. subtilis* BMV9. According to the classification proposed by Jitendra et al., these two biosurfactants can be identified as growth-associated metabolites, as there are clear correlations between bacterial growth, the utilization of available substrates and biosurfactant production [44]. Such growth-associated biosurfactant production has already been observed in other *Bacillus* species [45,46]. Similar patterns were found in all of the media tested with the exception of the 25% VJ medium, where the fermentation appeared to follow a different course, as lipopeptides continued to be produced in the initial stationary phase. An increase in biosurfactant levels in the stationary phase of growth is commonly associated with "production under growth-limiting conditions". This trend is quite common in *Pseudomonas* spp. [44,47] but has also been observed in *Bacillus subtilis* ATCC 21332 [48].

Focusing on the maximum concentrations of surfactin and fengycin produced in shake flasks, an increase in carbon substrates may have a positive effect on the production of growth-associated metabolites. However, in this study, increasing the percentage of VJ in the fermentation medium did not lead to an increase in lipopeptide production.

With regard to surfactin, similar maximum concentrations were found in the 25, 45 and 90% media (1.30, 1.36 and 1.31 g/L, respectively). The 70% VJ medium allowed *B. subtilis* BMV9 to produce greater quantities of this lipopeptide up to 1.64 g/L. However, comparing the surfactin values obtained with the different VJ-based media, the yields were comparable to those of the MSM medium (1.41 g/L). A similar trend was also observed for fengycin, with a slightly higher maximum concentration in 70% VJ medium (12.52 mg/L) and similar, slightly lower concentrations in media composed of 25% (11.00 mg/L), 45% (10.42 mg/L) and 90% (9.82 mg/L) VJ. As in the previous case, no significant differences were found between the fengycin concentrations in the media composed of food waste and the control medium (10.28 mg/L).

As can therefore be seen in Figure 1, in this study, there is no direct correlation between the increase in carbon substrates and the production of lipopeptides by *B. subtilis* BMV9.

Rather, there was a minimal, though not significative, increase in lipopeptide biosynthesis from 25% VJ to 70% and a slight decrease by increasing the percentage of VJ to 90%.

Over the years, several studies have attempted to investigate the effect of the initial concentration of glucose as a carbon source in the fermentation medium on biosurfactant production. For example, Hesty et al. studied the effect of the initial glucose concentration on surfactant production by *Bacillus* sp. BMN 14 and found that increasing the glucose concentration from 10 to 50 g/L did not result in a substantial increase in biosurfactants production. Furthermore, increasing the glucose concentration up to 70 g/L even led to a reduction in biosurfactant production, probably due to an osmotic-type phenomenon [49].

Moreover, Saúl et al. observed that increased glucose concentrations over 10.5 g/L did not lead to higher surfactin titers, suggesting that carbon-limited conditions may improve surfactin production abilities in *B. subtilis* cells [50]. Furthermore, it seems that the correlation between the increase in the carbonaceous substrate in the medium and the production of biosurfactants is dependent on the strain under investigation; indeed, Abushady et al. observed a linear increase in the surfactin concentration by increasing the initial glucose concentration in *B. subtilis* (BBk1) AB02238-1, but not in AB01335-1. In both cases, however, again, increasing the glucose concentration above 30 g/L did not lead to an improvement in lipopeptides production [47]. Yeh et al. hypothesized that glucose at certain concentrations may act as a limiting factor for surfactin production [51].

Shifting the focus to fengycin, Noomen et al. also found a limiting effect exerted by glucose on fengycin production, concluding that high glucose concentrations are not adequate for biosurfactant synthesis [52]. However, in the VJ media tested, another carbon source represented by fructose is present, which has been described as a substrate promoting fengycin synthesis in *Bacillus amyloliquefaciens* [53] but inhibiting it in *Bacillus circulans* [54]. Unfortunately, from our knowledge, no detailed studies are available to date on the influence of the initial concentration of various carbon substrates on the biosynthesis of fengycin and surfactin in *B. subtilis*.

The overview of the production parameters of the lipopeptides surfactin and fengycin by *B. subtilis* BMV9 in shake flask cultures using different media obtained from VJ is shown in Table 3.

Table 3. Comparison among media composed of different percentages of VJ in terms of the specific growth rate (μ), yield biomass/substrate ($Y_{x/s}$), yield product/substrate ($Y_{p/s}$), yield product/biomass ($Y_{p/x}$) and specific productivity product/biomass ($q_{p/x}$).

				Surfactin			Fengycin	
	μ (h^{-1})	**$Y_{x/s}$ (g/g)**	**$Y_{p/s}$ (g/g)**	**$Y_{p/x}$ (g/g)**	**$q_{p/x}$ (g/g·h)**	**$Y_{p/s}$ (mg/g)**	**$Y_{p/x}$ (mg/g)**	**$q_{p/x}$ (mg/g·h)**
MSM	0.193	0.188	0.046	0.263	0.009	0.369	1.886	0.079
90% VJ	0.172	0.075	0.011	0.184	0.006	0.080	1.580	0.033
70%VJ	0.170	0.085	0.021	0.249	0.01	0.126	2.209	0.046
45% VJ	0.206	0.088	0.021	0.323	0.01	0.253	2.288	0.095
25% VJ	0.368	0.240	0.054	0.568	0.02	0.457	4.809	0.200

Taking the media obtained from the food waste into consideration, a trend can be observed: as the percentage of VJ within the medium decreased, there was a progressive improvement in all calculated parameters.

There is a gradual increase in the biomass/substrate yield ($Y_{x/s}$) from 90% (0.075 g/g) to 25% (0.240 g/g). The same is also true for the product/biomass yield ($Y_{p/x}$): with a VJ percentage of 90% within the medium, the $Y_{p/x}$ value is 0.184 g/g for surfactin and 1.580 mg/g for fengycin; these values increased in fermentations conducted with media containing 70% (surfactin: 0.249 g/g; fengycin: 2.209 mg/g) and 45% (surfactin 0.323 g/g; fengycin 2.288 mg/g), reaching further increased values in the 25% VJ medium with $Y_{p/x}$

(surfactin) equal to 0.568 g/g and $Y_{p/x}$ (fengycin) equal to 4.809 mg/g. A similar trend also occurred in terms of the product/substrate yield. In media composed of 25% VJ, $Y_{p/s}$ is 0.054 g/g for surfactin and 0.457 mg/g for fengycin. These yield values decrease as the VJ within the medium increases, reaching values more than halved in 90% VJ (surfactin: 0.11 g/g; fengycin: 0.08 mg/g). The productivity $q_{p/x}$ was also higher in the 25% VJ medium than in all food waste media, with 0.02 g/g·h and 0.2 mg/g·h for surfactin and fengycin respectively.

Finally, the specific growth rate (μ) also followed the same trend; in fact, with the 25% VJ medium, this parameter was 0.386, which is higher than those calculated for the other media tested, with μ values of 0.206, 0.170 and 0.172 for the culture medium with VJ diluted to 45%, 70% and 90%, respectively.

On top of this, the results obtained with the 25% VJ medium were even better than those obtained with MSM (control).

The fact that better results were recorded in the 25% VJ medium in terms of both the yield and productivity is due to the fact that when microorganisms are limited in their energy source (usually the carbon source), high biomass yields are obtained as an effect of a closer correlation between catabolism and anabolism. In contrast, carbon excess-cultures generally have high carbon consumption rates and low biomass yields, as demonstrated by Dauner et al. [55]. Consequently, yield parameters that take into account bacterial biomass will be lower. Furthermore, Sheppard et al. noted an inverse correlation between biomass and surfactant production when the concentration of the carbon source was not restricted in *B. subtilis* culture. This would explain why as the availability of carbon substrates increases, there is no linear increase in the biosurfactants produced [56].

In light of these considerations, it was decided to use 25% VJ medium for the initial batch phase in the bioreactor scale-up.

3.2. Fed-Batch Bioreactor Cultivation

The use of fed-batch procedures offers distinct advantages over other fermentation strategies in bioreactors, such as continuous or batch processes [57]. For example, it has the possibility of producing high titers of lipopeptides in a short time, extending the log phase of growth. Moreover, these procedures facilitate enhanced control over nutrient availability, which can reduce the risk of the substrate.

The progress of the fed-batch fermentation conducted in a 10 L bioreactor using VJ as a carbon source is reported in Figure 2.

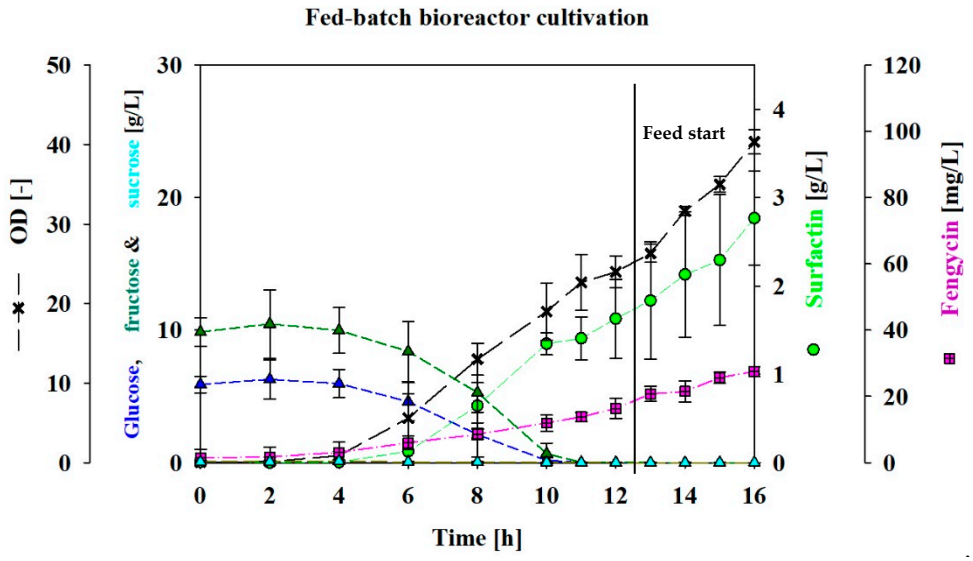

Figure 2. Fed-batch bioreactor cultivation pattern of *B. subtilis* BMV9 using vegetable juice in a 10L-Bioreactor.

In the initial batch phase, in which 25% VJ medium was used, *B. subtilis* BMV9 presented a shorter lag phase than that seen in the shake flask; in fact, already four hours after inoculation, the optical density value increased (OD_{600} 0.88), which indicated that the bacteria had entered the exponential phase of growth, as confirmed by the bacterial growth curve (Figure S2). The bacterium continued to replicate until the eleventh hour, reaching an OD_{600} of 22.67. Monosaccharides were also consumed from the fourth hour. In the literature, glucose is described as the preferred carbon and energy source of *B. subtilis* [58,59]; however, when this monosaccharide is co-present with other carbon sources within the same medium, *B. subtilis* does not always consume it first. On the one hand, Giro et al. reported that when both glucose and fructose are present, *B. subtilis* LAMI005 consumes glucose first [60]. On the other hand, Ponte Rocha et al. observed that *B. subtilis* LAMI008 preferentially consumes fructose first and glucose second in a medium consisting of Clarified Cashew Apple Juice [61]. In this study, however, the two monosaccharides showed the same consumption trend.

Surfactin and fengycin are also produced from the fourth hour onwards, reaching concentrations of 1.40 g/L and 13.79 mg/L, respectively, during the batch phase. At the eleventh hour from the start of fermentation, no residual substrates were detected, so at this point, the feed, consisting of the undiluted VJ, was pumped into the bioreactor. The result was an extension of lipopetides production. In particular, 5 h after the start of the feed, the optical density increased from 22.67 to 40.33. Similarly, the two lipopeptides continue to be produced by *B. subtilis* BMV9, reaching concentrations of 2.77 g/L for surfactin and 27.53 mg/L for fengycin at the 16th hour after bacterial inoculation. The production parameters of the lipopeptides surfactin and fengycin by *B. subtilis* BMV9 in fed-batch fermentation are shown in Table 4.

Table 4. Production parameters of fed-batch fermentation. Specific growth rate (μ), yield biomass/substrate ($Y_{x/s}$), yield product/substrate ($Y_{p/s}$), yield product/biomass ($Y_{p/x}$) and specific productivity are reported.

				Surfactin			**Fengycin**	
	μ (h^{-1})	**$Y_{x/s}$ (g/g)**	**$Y_{p/s}$ (g/g)**	**$Y_{p/x}$ (g/g)**	**$q_{p/x}$ (g/g·h)**	**$Y_{p/s}$ (mg/g)**	**$Y_{p/x}$ (mg/g)**	**$q_{p/x}$ (mg/g·h)**
Fed-batch fermentation	0.398	0.289	0.086	0.297	0.019	0.852	0.018	0.001

In the fed-batch cultivation, the bacterial strain exhibited a higher growth rate than that observed in the flask culture. In fact, the parameter $Y_{x/s}$, which indicates the product per substrate, increased. However, a substantial increase in bacterial biomass in the fed-batch fermentation contributed to the lowering of the calculated parameters $Y_{p/x}$ and $q_{p/x}$ concerning surfactin, which decreased from 0.568 g/g and 0.024 g/g·h in the flask to 0.297 g/g and 0.019 g/g·h in the bioreactor, respectively. The same happened for the parameters $Y_{p/x}$ and $q_{p/x}$ concerning fengycin: they went from 4.809 mg/g and 0.200 mg/g·h to 0.018 mg/g and 0.001 mg/g·h, respectively.

Although there was a decrease in yield parameters by performing the scale-up in the bioreactor, they still appear promising. For instance, Klausmann et al. performed a fed-batch fermentation on glucose and obtained a surfactin yield on biomass of 0.17 g/g using *B. subtilis* JABs24 and 0.23 g/g using *B. subtilis* JABs32, which is genetically similar to *B. subtilis* BMV9, but with an erythromycin resistance gene (*erm*). These parameters have been increased to 0.41 g/g and 0.57 g/g, respectively, by exploiting the two-step feed strategy [36].

It is interesting to see how the fed-batch strategy increased the $Y_{p/s}$ parameter to 0.086 g/g for surfactin and 0.852 mg/g, which means that by maintaining constant fermentation parameters, *B. subtilis* BMV9 was more efficient in utilizing substrates to biosynthesize both biosurfactants.

The $Y_{p/s}$ value of the lipopeptides in this study was higher than that reported by Coutte et al., who achieved a $Y_{fengycin/substrate}$ of 0.73 mg/g and a $Y_{surfactin/substrate}$ of 13.2 mg/g by performing batch fermentation on glucose in a bioreactor [62]. Moreover, Willennacher et al. obtained a surfactin/glucose yield of 0.05 g/g in the fed-batch strategy using *B. subtilis* DSM 10^T [43].

Through the fed-batch strategy, it was possible to significantly increase the production of the two lipopeptides of interest, even using a food waste medium. In particular, the amount of surfactin and fengycin was two- and threefold higher in the fed-batch cultivation in comparison to that in the shake flask cultivation (Figure 3).

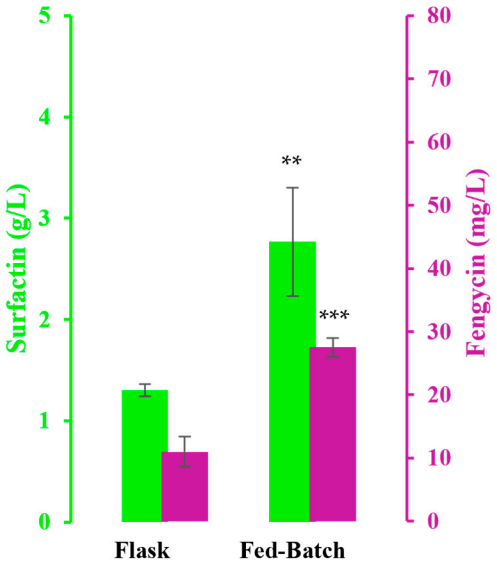

Figure 3. Comparison of maximum surfactin (g/L) and fengycin (mg/L) values produced by *B. subtilis* BMV9 in a shake flask using 25% VJ and fed-batch cultivation. Data of shake flasks cultivation are derived from one independent experiment in triplicate (n = 3); data from fed-batch cultivation are derived from three independent experiments (n = 3). Error bars represent SD. Asterisks indicate statistically different values in comparison to shake flask cultivation (*t*-test—**, $0.001 < p < 0.1$; ***, $p < 0.001$).

The fed-batch strategy had already been identified as an effective method for improving surfactin production [63]. Bouassida et al. showed that by exponential feeding in fed-batch cultivation, it is possible to increase the amount of lipopeptide produced by more than twofold compared to batch fermentation using *B. subtilis* SPB1 cultured in a glucose-based medium [64]; a similar result was found in the present study, in fact, with respect to the quantities of lipopeptides found in the flask cultivation (1.30 g/L of surfactin and 11.00 mg/L of fengycin produced in 25% VJ medium). *B. subtilis* BMV9 produced 2.77 g/L of surfactin and 27.53 mg/L of fengycin only 16 h after the start of fermentation in the bioreactor.

This result is quite interesting, as it demonstrates the possibility of obtaining similar quantities of lipopeptides as those obtained from waste media in other studies, but in a shorter time. For example, Nitschke et al. obtained 3 g/L of surfactin in 48 h from *B. subtilis* LB5a using cassava wastewater [41], while Ganesan et al. obtained 3.6 g/L and 3.65 g/L of surfactin after 24 and 68 h of incubation by culturing *B. subtilis* MTCC 2415 in grape juice and cashew apple juice, respectively [65].

Finally, Zhi et al. obtained similar amounts of surfactin (3.4 g/L) from distillery grains with co-cultures of *B. amyloliquefaciens* MT45 and X82 after 96 h of incubation [66].

The amount of fengycin produced in the bioreactor, although increased in comparison to flask cultivation, remains lower than in other studies, e.g., Geng-Rong et al. studied a co-culture system consisting of *B. subtilis* CGF-PG and *C. glu*-PR which, by fed-batch fermentation, produced 2309.96 mg/L fengycin in 96 h [67]; however, this result was not achieved in a waste medium.

In light of the results obtained, the question arises as to whether the industrial production of lipopeptides using food waste as an alternative carbon source is feasible. The most critical aspect to be considered is the possibility of obtaining a VJ that has the same sugars in terms of quality and quantity. This is very complex because the sugar composition of fruit and vegetables can vary depending on the harvest and seasonality; moreover, the juice that can be obtained from them can also change depending on the fruit and vegetables used. Therefore, to realize the scalability of the process on an industrial level, it would be necessary to standardize food waste. Indeed, any form of heterogeneity affects biomass growth, and it is a major concern when it comes to scaling up the process [68]. However, an effort in this direction is desirable, as the growing interest in obtaining biobased products from waste such as fruit and vegetables can not only improve waste management but also make food waste economically valuable [69,70]. Added to this is the possibility of reducing the production costs of lipopeptides. Despite the potential behind the microbial conversion of carbon sources into lipopeptides, it is necessary to further investigate the benefits and the economic–social implications it could exert on key stakeholders. In fact, these aspects have not been adequately investigated.

3.3. Surfactin and Fengycin Characterization through LC-MS/MS

Next, we asked the question of whether the use of a food waste medium affected the type and quantity of surfactin and fengycin lipopeptides produced by *B. subtilis* BMV9. Thus, LC-MS/MS analysis was performed for a detailed characterization of the lipopeptides produced from *B. subtilis* BMV9.

Bacteria of the genus Bacillus are capable of producing several surfactin congeners with fatty acid chains of 12 to 17 carbon atoms in length that could be linear, iso or anteiso branches and varying amino acid sequence [37,71,72]. So far, more than 40 different variants of surfactin have been identified [38]. In this study, LC-MS/MS analysis revealed that several surfactin variants in the range from m/z 944.636 to 1064.726 were produced under both growth conditions, 25% VJ and MSM supplemented with 20 g/L of glucose (Figure 4A,B).

In particular, the surfactin variants C12 at an m/z of 994.64, C13 at an m/z of 1008.66, C14 at an m/z of 1022.67, C15 at an m/z of 1036.69, C16 at an m/z of 1050.71 and C17 at an m/z of 1064.72 were observed. For both media, surfactin variants resulting from the substitution of amino acids in the major surfactin amino acid sequence (Glu-Leu-Leu-Val-Asp-Leu-Leu) at positions 2, 4 and 7 (Val2, Ala4 and Val7) and from the substitution at position 5 with the methyl ester of aspartate (AME5) were identified by the inspection of the MS/MS spectra. These amino acid modifications of the peptide moiety of the surfactin molecule produced by *Bacillus* spp. have already been described by Kecskemeti et al. and Vahidinasab et al. [37,73]. The relative quantification of surfactin variants was performed using extracted ion chromatograms (Figure S3A,B). For most of the surfactin variants, the percentage concentration was slightly higher in the 25% VJ medium than in the MSM (+20 g/L glucose) medium (Table S1).

Furthermore, fengycin lipopeptides could also be detected in MSM and 25% VJ samples by mass spectrometry (Figure 5A,B). The abundance of fengycin lipopeptides in the MSM and 25% VJ samples was approximately three orders of magnitude lower than that of surfactin lipopeptides, which is in line with the product/substrate yields determined in this study (Table 2). Fengycin produced by *Bacillus* spp. is a mixture of several homologues containing saturated or unsaturated fatty acid chains of different lengths and variants within the peptide moiety. Mainly, fengycin A and B with a saturated or unsaturated fatty acid chain with 14 to 18 carbon atoms were detected in both media conditions. Fengycin signals with a saturated fatty acid chain were detected at m/z = 1435.7 to 1519.9, and those with an unsaturated fatty acid chain were detected at m/z = 1433.8 to 1489.9, with the saturated variants being more abundant.

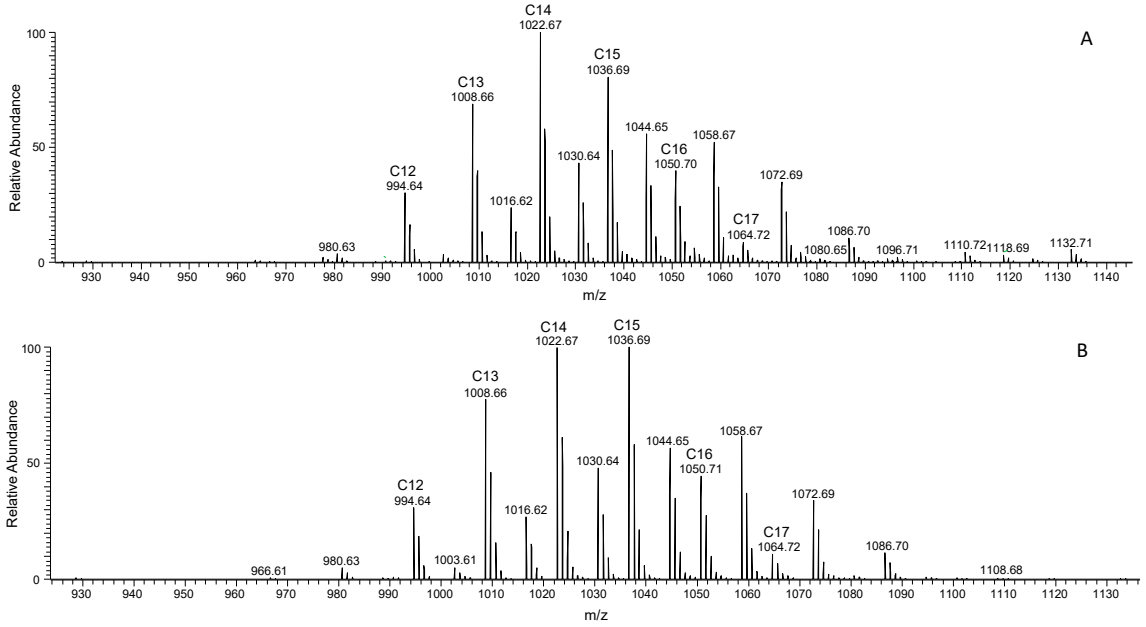

Figure 4. LC-MS spectra of surfactin lipopetides produced by *B. subtilis* BMV9 in 25% VJ (**A**) and in Mineral Salt Medium supplemented with 20 g/L glucose (**B**). The m/z values of protonated surfactin lipopeptides $[M + H]^+$ eluted in the time interval from 12 to 26 min are shown. Note that the MS spectrum shows the sum of all surfactin variants. A single m/z value may include several surfactin variants. The fatty acid chain lengths of different surfactin lipopeptides are indicated and refer to the major surfactin amino acid sequence (Glu-Leu-Leu-Val-Asp-Leu-Leu).

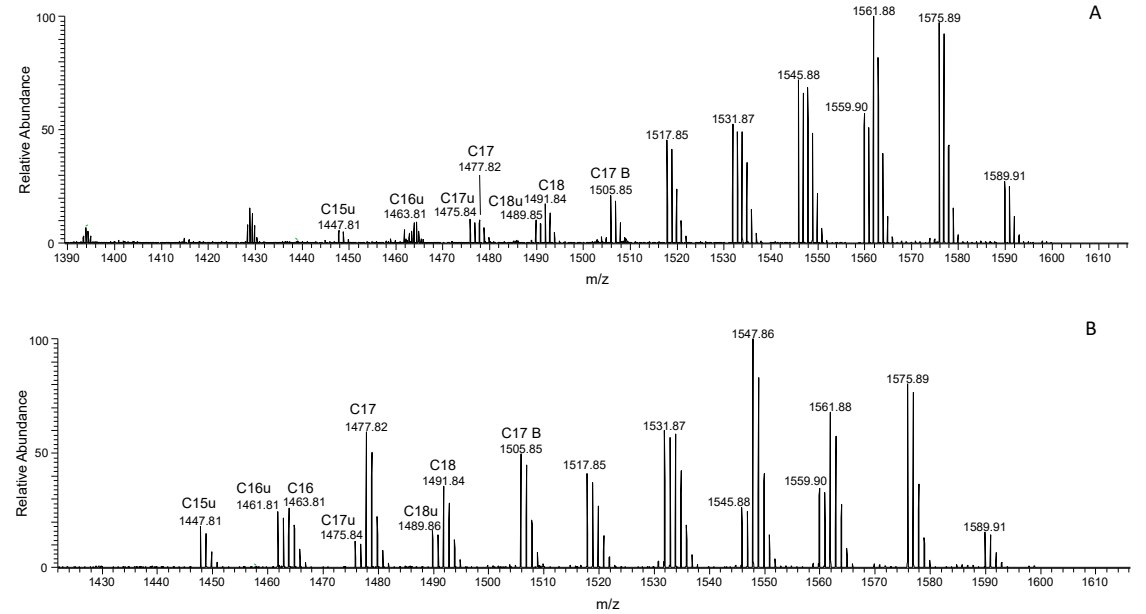

Figure 5. LC-MS spectra of fengycin lipopeptides produced by the *B. subtilis* BMV9 in 25% VJ (**A**) in Mineral Salt Medium supplemented with 20 g/L glucose (**B**). The m/z values of protonated fengycin lipopeptides $[M + H]^+$ eluted in the time interval from 2 to 10 min are shown. Fatty acid chain lengths of different fengycin lipopeptides are given. Note that the MS spectrum shows the sum of all fengycin variants, and that one m/z value may include more than one fengycin variant. The fatty acid chain lengths refer to the fengycin A variant unless otherwise stated.

Unfortunately, it was not possible to identify all fengycin variants, as the intensity of the precursor ions was too low to generate meaningful fragmentation spectra. Therefore, the annotation of fengycin lipopeptides was performed based on the results of previous

experiments [37]. However, looking at the two MS spectra (Figure 5A,B), there is a tendency for higher m/z values of fengycin lipopeptides in the presence of vegetable waste juice than in the MSM medium, in contrast to the surfactin lipopeptides, which showed a very similar distribution of m/z values regardless of the carbon source used. Higher m/z values may be associated with lipopeptide variants with longer fatty acid chains or a different amino acid composition.

4. Conclusions

This study demonstrated that VJ can be used as an alternative carbon source for the production of lipopeptides such as surfactin and fengycin by *Bacillus subtilis* BMV9. The results of this study show that *B. subtilis* BMV9 can utilize VJ in different levels of concentration, with an impact on lipopeptide production. Shake flask cultivation experiments revealed that surfactin and fengycin production using VJ is feasible, thus replacing conventional glucose-based media, offering an inexpensive alternative for industrial bioprocesses.

Fed-batch bioreactor cultivations further optimized the production bioprocess, achieving higher concentrations of surfactin and fengycin compared to flask cultivations. The switch from stirring flasks to a 10 L bioreactor underlines the potential for industrial application, with the constant control of growth parameters ensuring reliable and higher production. Furthermore, the application of LC-MS for the characterization of surfactin and fengycin provided a detailed molecular understanding of the bioproducts, confirming that surfactin produced from VJ is comparable to that produced using glucose-based media, while fengycin, interestingly, has higher m/z values when produced from VJ.

In conclusion, this study contributes to the field of microbial biotechnology by presenting a process for the production of valuable bio-compounds, and, at the same time, the adoption of VJ as a sustainable carbon source for the production of secondary metabolites such as lipopeptides significantly contributes to environmental sustainability. This strategy aligns with global efforts to reduce greenhouse gas emissions associated with food waste and the disposal of organic materials in landfills, thereby supporting the transition towards a more sustainable and circular bioeconomy and upcycling.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10060323/s1, Figure S1: Growth curve of *B. subtilis* BMV9 growing on media composed of different percentages of VJ and on MSM + 20 g/L glucose. The bacterial growth curve over time is represented in a semilogarithmic plot. Figure S2: Growth curve of *B. subtilis* BMV9 during fed-batch cultivation using vegetable juice in a 10 L bioreactor. The bacterial growth curve over time is represented in a semi-logarithmic plot. Figure S3: Extracted ion chromatograms of surfactin lipopeptides in 25% VJ (A) and in Mineral Salt Medium supplemented with 20 g/L glucose (B). Table S1: Surfactin variants detected by LC-MS analysis in 25% VJ and MSM added with 20 g/L of glucose after 24 h of *B. subtilis* BMV9 shake flask cultivation. Precursor ions (M + H)+, retention time, diagnostic fragment ions and corresponding surfactin variants are shown.

Author Contributions: Conceptualization, S.M., S.C., E.H.B.P., S.V. and M.V.; methodology, I.G., M.V., E.H.B.P. and E.H.; software, I.G.; validation, I.G., M.V. and E.H.B.P.; formal analysis, I.G., E.H.B.P., J.P., M.V. and P.K.; investigation, I.G. and F.M.; resources, E.H.B.P. and M.V.; data curation, M.V. and E.H.B.P.; writing—original draft preparation, I.G.; writing—review and editing, S.C., S.M., R.H., M.V. and E.H.B.P.; visualization, I.G. and M.V.; supervision, R.H., S.M. and S.V.; project administration, M.V. and R.H.; funding acquisition, S.M., S.V. and S.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by S.C., who is a researcher funded by the European Union Grant—FSE REACT-EU, PON Research and Innovation 2014–2020 Grant n. 2021: PON-DM-1062-SC-RIC CUP F71B21005820007 to S.M.; Irene Gugel's PhD position is supported by the Ministry of University and Research Grant n. DOT1309055 to S.V. The project was funded (S.M.) under the National Recovery and Resilience Plan (NRRP), Mission 04 Component 2 Investment 1.5—NextGenerationEU, call for tender n. 3277 dated 30 December 2021 Award Number: 0001052 dated 23 June 2022 and Ambrosialab (Horizon 2020 research and innovation program under grant agreement SbD4Nano, No. 862195; Horizon Europe, under grant agreement SUNRISE No. 101137324).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Acknowledgments: S.M. wishes to thank Elisa Durini for her helpful technical support, Ambrosialab (Italy) for the kind donation of materials used for the experiments and Neotron Spa (Italy) for their assistance with the VJ characterization analysis.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study, in the collection, analyses or interpretation of the data, in the writing of the manuscript or in the decision to publish the results.

References

1. Kaur, J.; Rani, G.; Yogalakshmi, K. Problems and Issues of Food Waste-Based Biorefineries. In *Food Waste to Valuable Resources*; Elsevier: Amsterdam, The Netherlands, 2020; pp. 343–357.
2. D'Adamo, I.; Desideri, S.; Gastaldi, M.; Tsagarakis, K.P. Sustainable Food Waste Management in Supermarkets. *Sustain. Prod. Consum.* **2023**, *43*, 204–216. [CrossRef]
3. Melikoglu, M.; Lin, C.S.K.; Webb, C. Analysing Global Food Waste Problem: Pinpointing the Facts and Estimating the Energy Content. *Cent. Eur. J. Eng.* **2013**, *3*, 157–164. [CrossRef]
4. Bigdeloo, M.; Teymourian, T.; Kowsari, E.; Ramakrishna, S.; Ehsani, A. Sustainability and Circular Economy of Food Wastes: Waste Reduction Strategies, Higher Recycling Methods, and Improved Valorization. *Mater. Circ. Econ.* **2021**, *3*, 3. [CrossRef]
5. Rohini, C.; Geetha, P.; Vijayalakshmi, R.; Mini, M.; Pasupathi, E. Global Effects of Food Waste. *J. Pharmacogn. Phytochem.* **2020**, *9*, 690–699.
6. Jones, R.; Speight, R.; Blinco, J.; O'Hara, I. Biorefining within Food Loss and Waste Frameworks: A Review. *Renew. Sustain. Energy Rev.* **2022**, *154*, 111781. [CrossRef]
7. UNEP DTU Partnership and United Nations Environment Programme. *Reducing Consumer Food Waste Using Green and Digital Technologies*; UNEP DTU Partnership: Copenhagen, Denmark; Nairobi, Kenya, 2021.
8. De Laurentiis, V.; Mancini, L.; Casonato, C.; Boysen-Urban, K.; De Jong, B.; M'Barek, R.; Sanyé Mengual, E.; Sala, S. Setting the Scene for an EU Initiative on Food Waste Reduction Targets. *Publ. Off. Eur. Union Luxemb. Doi* **2023**, *10*, 13859.
9. Scialabba, N.E. *Food Wastage Footprint Full-Cost Accounting: Final Report*; FAO: Rome, Italy, 2014; ISBN 978-92-5-108512-7. Available online: http://www.fao.org/3/a-i3991e.pdf (accessed on 6 October 2020).
10. Brancoli, P.; Rousta, K.; Bolton, K. Life Cycle Assessment of Supermarket Food Waste. *Resour. Conserv. Recycl.* **2017**, *118*, 39–46. [CrossRef]
11. Mattsson, L.; Williams, H.; Berghel, J. Waste of Fresh Fruit and Vegetables at Retailers in Sweden–Measuring and Calculation of Mass, Economic Cost and Climate Impact. *Resour. Conserv. Recycl.* **2018**, *130*, 118–126. [CrossRef]
12. Eriksson, M.; Strid, I.; Hansson, P.-A. Food Losses in Six Swedish Retail Stores: Wastage of Fruit and Vegetables in Relation to Quantities Delivered. *Resour. Conserv. Recycl.* **2012**, *68*, 14–20. [CrossRef]
13. Scholz, K.; Eriksson, M.; Strid, I. Carbon Footprint of Supermarket Food Waste. *Resour. Conserv. Recycl.* **2015**, *94*, 56–65. [CrossRef]
14. Papargyropoulou, E.; Lozano, R.; Steinberger, J.K.; Wright, N.; bin Ujang, Z. The Food Waste Hierarchy as a Framework for the Management of Food Surplus and Food Waste. *J. Clean. Prod.* **2014**, *76*, 106–115. [CrossRef]
15. Liakou, V.; Pateraki, C.; Palaiogeorgou, A.-M.; Kopsahelis, N.; de Castro, A.M.; Freire, D.M.G.; Nychas, G.-J.E.; Papanikolaou, S.; Koutinas, A. Valorisation of Fruit and Vegetable Waste from Open Markets for the Production of 2, 3-Butanediol. *Food Bioprod. Process.* **2018**, *108*, 27–36. [CrossRef]
16. Mitrea, L.; Calinoiu, L.F.; Teleky, B.E.; Szabo, K.; Martău, A.G.; Nemes, S.A.; Plamada, D.; Pascuta, M.S.; Barta, G.; Varvara, R.A. Fruit and Vegetable Wastes for Biobased Chemicals. In *Fruit and Vegetable Waste Utilization and Sustainability*; Elsevier: Amsterdam, The Netherlands, 2023; pp. 43–76.
17. Dahiya, S.; Kumar, A.N.; Sravan, J.S.; Chatterjee, S.; Sarkar, O.; Mohan, S.V. Food Waste Biorefinery: Sustainable Strategy for Circular Bioeconomy. *Bioresour. Technol.* **2018**, *248*, 2–12. [CrossRef] [PubMed]
18. Magama, P.; Chiyanzu, I.; Mulopo, J. A Systematic Review of Sustainable Fruit and Vegetable Waste Recycling Alternatives and Possibilities for Anaerobic Biorefinery. *Bioresour. Technol. Rep.* **2022**, *18*, 101031. [CrossRef]
19. Nagarajan, D.; Lee, D.-J.; Chang, J.-S. Circular Bioeconomy: An Introduction. In *Biomass, Biofuels, Biochemicals*; Elsevier: Amsterdam, The Netherlands, 2021; pp. 3–23.
20. Tan, E.C.; Lamers, P. Circular Bioeconomy Concepts—A Perspective. *Front. Sustain.* **2021**, *2*, 701509. [CrossRef]

21. Gaur, V.K.; Sharma, P.; Sirohi, R.; Varjani, S.; Taherzadeh, M.J.; Chang, J.-S.; Ng, H.Y.; Wong, J.W.; Kim, S.-H. Production of Biosurfactants from Agro-Industrial Waste and Waste Cooking Oil in a Circular Bioeconomy: An Overview. *Bioresour. Technol.* **2022**, *343*, 126059. [CrossRef] [PubMed]
22. Mohanty, S.S.; Koul, Y.; Varjani, S.; Pandey, A.; Ngo, H.H.; Chang, J.-S.; Wong, J.W.; Bui, X.-T. A Critical Review on Various Feedstocks as Sustainable Substrates for Biosurfactants Production: A Way towards Cleaner Production. *Microb. Cell Factories* **2021**, *20*, 120. [CrossRef] [PubMed]
23. Zhu, Z.; Zhang, B.; Cai, Q.; Ling, J.; Lee, K.; Chen, B. Fish Waste Based Lipopeptide Production and the Potential Application as a Bio-Dispersant for Oil Spill Control. *Front. Bioeng. Biotechnol.* **2020**, *8*, 734. [CrossRef] [PubMed]
24. Wang, Z.; Liu, C.; Shi, Y.; Huang, M.; Song, Z.; Simal-Gandara, J.; Li, N.; Shi, J. Classification, Application, Multifarious Activities and Production Improvement of Lipopeptides Produced by Bacillus. *Crit. Rev. Food Sci. Nutr.* **2023**, 1–14. [CrossRef]
25. Hamdache, A.; Azarken, R.; Lamarti, A.; Aleu, J.; Collado, I.G. Comparative Genome Analysis of Bacillus spp. and Its Relationship with Bioactive Nonribosomal Peptide Production. *Phytochem. Rev.* **2013**, *12*, 685–716. [CrossRef]
26. Zhao, H.; Shao, D.; Jiang, C.; Shi, J.; Li, Q.; Huang, Q.; Rajoka, M.S.R.; Yang, H.; Jin, M. Biological Activity of Lipopeptides from *Bacillus*. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 5951–5960. [CrossRef] [PubMed]
27. Kanlayavattanakul, M.; Lourith, N. Lipopeptides in Cosmetics. *Int. J. Cosmet. Sci.* **2010**, *32*, 1–8. [CrossRef] [PubMed]
28. Al-Bahry, S.; Al-Wahaibi, Y.; Elshafie, A.; Al-Bemani, A.; Joshi, S.; Al-Makhmari, H.; Al-Sulaimani, H. Biosurfactant Production by *Bacillus subtilis* B20 Using Date Molasses and Its Possible Application in Enhanced Oil Recovery. *Int. Biodeterior. Biodegrad.* **2013**, *81*, 141–146. [CrossRef]
29. Umar, A.; Zafar, A.; Wali, H.; Siddique, M.P.; Qazi, M.A.; Naeem, A.H.; Malik, Z.A.; Ahmed, S. Low-Cost Production and Application of Lipopeptide for Bioremediation and Plant Growth by *Bacillus subtilis* SNW3. *AMB Express* **2021**, *11*, 165. [CrossRef] [PubMed]
30. Moshtagh, B.; Hawboldt, K.; Zhang, B. Optimization of Biosurfactant Production by *Bacillus subtilis* N3-1P Using the Brewery Waste as the Carbon Source. *Environ. Technol.* **2018**, *40*, 3371–3380. [CrossRef] [PubMed]
31. Gudiña, E.J.; Fernandes, E.C.; Rodrigues, A.I.; Teixeira, J.A.; Rodrigues, L.R. Biosurfactant Production by *Bacillus subtilis* Using Corn Steep Liquor as Culture Medium. *Front. Microbiol.* **2015**, *6*, 126037.
32. Herrmann, L.W.; Letti, L.A.J.; de Oliveira Penha, R.; Soccol, V.T.; Rodrigues, C.; Soccol, C.R. *Bacillus* Genus Industrial Applications and Innovation: First Steps towards a Circular Bioeconomy. *Biotechnol. Adv.* **2023**, *70*, 108300. [CrossRef] [PubMed]
33. Vahidinasab, M.; Lilge, L.; Reinfurt, A.; Pfannstiel, J.; Henkel, M.; Morabbi Heravi, K.; Hausmann, R. Construction and Description of a Constitutive Plipastatin Mono-Producing *Bacillus subtilis*. *Microb. Cell Factories* **2020**, *19*, 205. [CrossRef] [PubMed]
34. Reuß, D.R.; Thürmer, A.; Daniel, R.; Quax, W.J.; Stülke, J. Complete Genome Sequence of *Bacillus subtilis* Subsp. *subtilis* StrainΔ 6. *Genome Announc.* **2016**, *4*, 10–1128.
35. Wenzel, M.; Müller, A.; Siemann-Herzberg, M.; Altenbuchner, J. Self-Inducible *Bacillus subtilis* Expression System for Reliable and Inexpensive Protein Production by High-Cell-Density Fermentation. *Appl. Environ. Microbiol.* **2011**, *77*, 6419–6425. [CrossRef]
36. Klausmann, P.; Hennemann, K.; Hoffmann, M.; Treinen, C.; Aschern, M.; Lilge, L.; Morabbi Heravi, K.; Henkel, M.; Hausmann, R. *Bacillus subtilis* High Cell Density Fermentation Using a Sporulation-Deficient Strain for the Production of Surfactin. *Appl. Microbiol. Biotechnol.* **2021**, *105*, 4141–4151. [CrossRef] [PubMed]
37. Vahidinasab, M.; Adiek, I.; Hosseini, B.; Akintayo, S.O.; Abrishamchi, B.; Pfannstiel, J.; Henkel, M.; Lilge, L.; Voegele, R.T.; Hausmann, R. Characterization of *Bacillus velezensis* UTB96, Demonstrating Improved Lipopeptide Production Compared to the Strain *B. velezensis* FZB42. *Microorganisms* **2022**, *10*, 2225. [CrossRef] [PubMed]
38. Geissler, M.; Oellig, C.; Moss, K.; Schwack, W.; Henkel, M.; Hausmann, R. High-Performance Thin-Layer Chromatography (HPTLC) for the Simultaneous Quantification of the Cyclic Lipopeptides Surfactin, Iturin A and Fengycin in Culture Samples of *Bacillus* Species. *J. Chromatogr. B* **2017**, *1044*, 214–224. [CrossRef]
39. Hoffmann, M.; Fernandez Cano Luna, D.S.; Xiao, S.; Stegemüller, L.; Rief, K.; Heravi, K.M.; Lilge, L.; Henkel, M.; Hausmann, R. Towards the Anaerobic Production of Surfactin Using *Bacillus subtilis*. *Front. Bioeng. Biotechnol.* **2020**, *8*, 554903. [CrossRef] [PubMed]
40. Sandrin, C.; Peypoux, F.; Michel, G. Coproduction of surfactin and iturin A, lipopeptides with surfactant and antifungal properties, by *Bacillus subtilis*. *Biotechnol. Appl. Biochem.* **1990**, *12*, 370–375. [CrossRef] [PubMed]
41. Nitschke, M.; Pastore, G.M. Production and Properties of a Surfactant Obtained from *Bacillus subtilis* Grown on Cassava Wastewater. *Bioresour. Technol.* **2006**, *97*, 336–341. [CrossRef] [PubMed]
42. de Oliveira Schmidt, V.K.; Moraes, P.A.D.; Cesca, K.; Pereira, L.P.S.; de Andrade, L.M.; Mendes, M.A.; de Oliveira, D.; de Andrade, C.J. Enhanced Production of Surfactin Using Cassava Wastewater and Hydrophobic Inducers: A Prospection on New Homologues. *World J. Microbiol. Biotechnol.* **2023**, *39*, 82. [CrossRef] [PubMed]
43. Willenbacher, J.; Yeremchuk, W.; Mohr, T.; Syldatk, C.; Hausmann, R. Enhancement of Surfactin Yield by Improving the Medium Composition and Fermentation Process. *Amb Express* **2015**, *5*, 57. [CrossRef] [PubMed]
44. Desai, J.D.; Banat, I.M. Microbial Production of Surfactants and Their Commercial Potential. *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 47–64.

45. Wei, Y.; Wang, L.; Chang, J. Optimizing Iron Supplement Strategies for Enhanced Surfactin Production with *Bacillus subtilis*. *Biotechnol. Prog.* **2004**, *20*, 979–983. [CrossRef]
46. Kim, H.-S.; Yoon, B.-D.; Lee, C.-H.; Suh, H.-H.; Oh, H.-M.; Katsuragi, T.; Tani, Y. Production and Properties of a Lipopeptide Biosurfactant from *Bacillus subtilis* C9. *J. Ferment. Bioeng.* **1997**, *84*, 41–46. [CrossRef]
47. Abushady, H.; Bashandy, A.; Aziz, N.; Ibrahim, H. Molecular Characterization of *Bacillus subtilis* Surfactin Producing Strain and the Factors Affecting Its Production. *Int. J. Agric. Biol.* **2005**, *7*, 337–344.
48. Davis, D.; Lynch, H.; Varley, J. The Production of Surfactin in Batch Culture by *Bacillus subtilis* ATCC 21332 Is Strongly Influenced by the Conditions of Nitrogen Metabolism. *Enzyme Microb. Technol.* **1999**, *25*, 322–329. [CrossRef]
49. Heryani, H.; Putra, M.D. Kinetic Study and Modeling of Biosurfactant Production Using *Bacillus* Sp. *Electron. J. Biotechnol.* **2017**, *27*, 49–54. [CrossRef]
50. Alonso, S.; Martin, P.J. Impact of Foaming on Surfactin Production by *Bacillus subtilis*: Implications on the Development of Integrated in Situ Foam Fractionation Removal Systems. *Biochem. Eng. J.* **2016**, *110*, 125–133. [CrossRef]
51. Yeh, M.; Wei, Y.; Chang, J. Enhanced Production of Surfactin from *Bacillus subtilis* by Addition of Solid Carriers. *Biotechnol. Prog.* **2005**, *21*, 1329–1334. [CrossRef] [PubMed]
52. Hmidet, N.; Ben Ayed, H.; Jacques, P.; Nasri, M. Enhancement of Surfactin and Fengycin Production by *Bacillus mojavensis* A21: Application for Diesel Biodegradation. *BioMed Res. Int.* **2017**, *2017*, 5893123. [CrossRef] [PubMed]
53. Lu, H.; Qian, S.; Muhammad, U.; Jiang, X.; Han, J.; Lu, Z. Effect of Fructose on Promoting Fengycin Biosynthesis in *Bacillus amyloliquefaciens* Fmb-60. *J. Appl. Microbiol.* **2016**, *121*, 1653–1664. [CrossRef]
54. van Niekerk, K.; Pott, R.W. The Effect of Carbon Source on the Growth and Lipopeptide Production of *Bacillus circulans*. *Biocatal. Agric. Biotechnol.* **2023**, *53*, 102841. [CrossRef]
55. Dauner, M.; Storni, T.; Sauer, U. *Bacillus subtilis* Metabolism and Energetics in Carbon-Limited and Excess-Carbon Chemostat Culture. *J. Bacteriol.* **2001**, *183*, 24. [CrossRef]
56. Sheppard, J.D.; Mulligan, C.N. The Production of Surfactin by *Bacillus subtilis* Grown on Peat Hydrolysate. *Appl. Microbiol. Biotechnol.* **1987**, *27*, 110–116. [CrossRef]
57. Minihane, B.; Brown, D. Fed-Batch Culture Technology. *Biotechnol. Adv.* **1986**, *4*, 207–218. [CrossRef] [PubMed]
58. Stülke, J.; Hillen, W. Regulation of Carbon Catabolism in *Bacillus* Species. *Annu. Rev. Microbiol.* **2000**, *54*, 849–880. [CrossRef] [PubMed]
59. Buffing, M.F.; Link, H.; Christodoulou, D.; Sauer, U. Capacity for Instantaneous Catabolism of Preferred and Non-Preferred Carbon Sources in *Escherichia coli* and *Bacillus subtilis*. *Sci. Rep.* **2018**, *8*, 11760. [CrossRef] [PubMed]
60. Giro, M.E.A.; Martins, J.J.L.; Rocha, M.V.P.; Melo, V.M.M.; Gonçalves, L.R.B. Clarified Cashew Apple Juice as Alternative Raw Material for Biosurfactant Production by *Bacillus subtilis* in a Batch Bioreactor. *Biotechnol. J.* **2009**, *4*, 738–747. [CrossRef] [PubMed]
61. Ponte Rocha, M.V.; Gomes Barreto, R.V.; Melo, V.M.M.; Barros Gonçalves, L.R. Evaluation of Cashew Apple Juice for Surfactin Production by *Bacillus subtilis* LAMI008. *Appl. Biochem. Biotechnol.* **2009**, *155*, 63–75. [CrossRef] [PubMed]
62. Coutte, F.; Lecouturier, D.; Ait Yahia, S.; Leclère, V.; Béchet, M.; Jacques, P.; Dhulster, P. Production of Surfactin and Fengycin by *Bacillus subtilis* in a Bubbleless Membrane Bioreactor. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 499–507. [CrossRef]
63. Amin, G. Exponential Fed-Batch Strategy for Enhancing Biosurfactant Production by *Bacillus subtilis*. *Water Sci. Technol.* **2014**, *70*, 234–240. [CrossRef] [PubMed]
64. Bouassida, M.; Mnif, I.; Ghribi, D. Enhanced Biosurfactant Production by *Bacillus subtilis* SPB1 Using Developed Fed-Batch Fermentation: Effects of Glucose Levels and Feeding Systems. *Bioprocess Biosyst. Eng.* **2023**, *46*, 555–563. [CrossRef]
65. Ganesan, N.G.; Rangarajan, V. A Comparative Study on Surfactin Production from Various Fruit Juices for Diverse Applications. *Mater. Today Proc.* **2023**, *72*, 2660–2667. [CrossRef]
66. Zhi, Y.; Wu, Q.; Xu, Y. Production of Surfactin from Waste Distillers' Grains by Co-Culture Fermentation of Two *Bacillus amyloliquefaciens* Strains. *Bioresour. Technol.* **2017**, *235*, 96–103. [CrossRef] [PubMed]
67. Gao, G.-R.; Wei, S.-Y.; Ding, M.-Z.; Hou, Z.-J.; Wang, D.-J.; Xu, Q.-M.; Cheng, J.-S.; Yuan, Y.-J. Enhancing Fengycin Production in the Co-Culture of *Bacillus subtilis* and *Corynebacterium glutamicum* by Engineering Proline Transporter. *Bioresour. Technol.* **2023**, *383*, 129229. [CrossRef]
68. Hewitt, C.J.; Nienow, A.W. The Scale-up of Microbial Batch and Fed-batch Fermentation Processes. *Adv. Appl. Microbiol.* **2007**, *62*, 105–135. [PubMed]
69. Bhatia, L.; Jha, H.; Sarkar, T.; Sarangi, P.K. Food Waste Utilization for Reducing Carbon Footprints towards Sustainable and Cleaner Environment: A Review. *Int. J. Environ. Res. Public Health* **2023**, *20*, 2318. [CrossRef]
70. Adamu, H.; Bello, U.; Yuguda, A.U.; Tafida, U.I.; Jalam, A.M.; Sabo, A.; Qamar, M. Production Processes, Techno-Economic and Policy Challenges of Bioenergy Production from Fruit and Vegetable Wastes. *Renew. Sustain. Energy Rev.* **2023**, *186*, 113686. [CrossRef]
71. Hoffmann, M.; Mück, D.; Grossmann, L.; Greiner, L.; Klausmann, P.; Henkel, M.; Lilge, L.; Weiss, J.; Hausmann, R. Surfactin from *Bacillus subtilis* Displays Promising Characteristics as O/W-Emulsifier for Food Formulations. *Colloids Surf. B Biointerfaces* **2021**, *203*, 111749. [CrossRef] [PubMed]

72. Théatre, A.; Cano-Prieto, C.; Bartolini, M.; Laurin, Y.; Deleu, M.; Niehren, J.; Fida, T.; Gerbinet, S.; Alanjary, M.; Medema, M.H. The Surfactin-like Lipopeptides from *Bacillus* spp.: Natural Biodiversity and Synthetic Biology for a Broader Application Range. *Front. Bioeng. Biotechnol.* **2021**, *9*, 623701. [CrossRef]
73. Kecskeméti, A.; Bartal, A.; Bóka, B.; Kredics, L.; Manczinger, L.; Shine, K.; Alharby, N.S.; Khaled, J.M.; Varga, M.; Vágvölgyi, C. High-Frequency Occurrence of Surfactin Monomethyl Isoforms in the Ferment Broth of a *Bacillus subtilis* Strain Revealed by Ion Trap Mass Spectrometry. *Molecules* **2018**, *23*, 2224. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.